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Published in:
Insect Biochemistry and Molecular Biology

DOI:
10.1016/j.ibmb.2017.09.007

Insect Biochemistry and Molecular Biology 90 (2017) 23–33

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Maternal provision of *transformer-2* is required for female development and embryo viability in the wasp *Nasonia vitripennis*

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ARTICLE INFO

Article history:
Received 20 April 2017
Received in revised form
8 September 2017
Accepted 13 September 2017
Available online 18 September 2017

Keywords:
Sex determination
RNA interference
Transformer-2
Transformer
Nasonia vitripennis
Diploid males

ABSTRACT

In insect sex determination a primary signal starts the genetic sex determination cascade that, in most insect orders, is subsequently transduced down the cascade by a *transformer* (*tra*) ortholog. Only a female-specifically spliced *tra* mRNA yields a functional TRA-protein that forms a complex with TRA2, encoded by a *transformer-2* (*tra2*) ortholog, to act as a sex specific splicing regulator of the downstream transcription factors *doublesex* (*dsx*) and *fruitless* (*fru*). Here, we identify the *tra2* ortholog of the haplodiploid parasitoid wasp *N. vitripennis* (*Nv-tra2*) and confirm its function in *N. vitripennis* sex determination. Knock down of *Nv-tra2* by parental RNA interference (pRNAi) results in complete sex reversal of diploid offspring from female to male, indicating the requirement of *Nv-tra2* for female sex determination. As *Nv-tra2* pRNAi leads to frequent lethality in early developmental stages, maternal provision of *Nv-tra2* transcripts is apparently also required for another, non-sex determining function during embryogenesis. In addition, lethality following *Nv-tra2* pRNAi appears more pronounced in diploid than in haploid offspring. This diploid lethal effect was also observed following *Nv-tra* pRNAi, which served as a positive control in our experiments. As diploid embryos from fertilized eggs have a paternal chromosome set in addition to the maternal one, this suggests that either the presence of this paternal chromosome set or the dosage effect resulting from the diploid state is incompatible with the induced male development in *N. vitripennis* caused by either *Nv-tra2* or *Nv-tra* pRNAi. The role of *Nv-tra2* in activating the female sex determination pathway yields more insight into the sex determination mechanism of *Nasonia*.

1. Introduction

Insect sex determination involves a cascade of regulatory genes that is evolving bottom-up (Wilkins, 1995). *Doublesex* (*dsx*), the interpreter for the sexual identity of the cell, is the most conserved gene at the bottom of the cascade (Shukla and Nagaraju, 2010; Verhulst and van de Zande, 2015). The primary signal at the top of the cascade directs sex determination into the female or male mode and is highly variable throughout the insect class (Sánchez, 2008). The regulation of sex determination resembles an hourglass model in which the variable primary signal is considered as the instruction, and the multiple actions of *dsx* as the execution phase (Bopp et al., 2014). In most insects, the center of the hourglass is represented by *transformer* (*tra*), a fast-evolving gene that transduces the primary signal through sex-specific splicing (Sánchez, 2008; Verhulst et al., 2010b). Only when the *tra* transcript is spliced in the female-variant, it can be translated into a functional TRA protein that will regulate female specific splicing of *dsx* transcripts. In addition, *tra* maintains its own female-specific splicing mode through an auto-regulatory loop that functions as a "memory" to ensure proper sexual differentiation (Bopp et al., 2014).

Transformer-2 (TRA2) is an essential co-factor in the sex determination of many, if not all, insect species (Amrein et al., 1990; Hedley and Maniatis, 1991; Inoue et al., 1992; Salvemini et al., 2009; Hediger et al., 2010; Schetelig et al., 2012). Knockdown studies of *tra2* in Diptera revealed the involvement of TRA2 in the female-specific splicing of *tra* mRNAs (Burghardt et al., 2005; Concha and Scott, 2009; Martín et al., 2011; Salvemini et al.,

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http://dx.doi.org/10.1016/j.ibmb.2017.09.007

2009; Sarno et al., 2010). In some insects species TRA2 has alternative isoforms which all code for a basic RNA-binding domain (RBD), but none of these isoforms are sex-specific at any life stage (Martín et al., 2011; Nissen et al., 2012; Niu et al., 2005; Shukla and Palli, 2013). In other insect species only one TRA2 isoform was detected (Burghardt et al., 2005; Concha and Scott, 2009; Liu et al., 2015; Salvemini et al., 2009; Sarno et al., 2010; Schetelig et al., 2012). *Tra2* is more conserved than *tra*, as even in species without a *tra* ortholog, like the lepidopteran *Bombyx mori*, a *tra2* homolog was identified (Kiuchi et al., 2014; Niu et al., 2005).

In Hymenoptera that have a haplodiploid reproductive system, males develop from haploid unfertilized eggs and females from diploid fertilized eggs. Two mechanisms of sex determination have thus far functionally been described: the Complementary Sex Determination (CSD) in *Apis mellifera* and the Maternal Effect Genomic Imprinting Sex Determination (MEGISD) in *Nasonia vitripennis*. CSD involves the complementation of alleles at the *csd* locus, in which heterozygosity at one or more *csd* loci leads to female development (Beye et al., 2003; Cook, 1993; van Wilgenburg et al., 2006). Its genetic basis has only been elucidated for the honeybee (*Apis mellifera*), where the *csd* locus was identified as a duplication of *feminizer* (*fem*), the honeybee ortholog of *tra* (Beye et al., 2003; Hasselmann et al., 2008; Gempe et al., 2009). The MEGISD model (Beukeboom et al., 2007; Beukeboom and van de Zande, 2010) involves maternal imprinting, maternal provision of *tra* mRNA and activation of zygotic *tra* transcription (Verhulst et al., 2010a, 2013). In this model the non-silenced paternal allele of the hypothetical *womanizer (wom)* gene will initiate zygotic transcription of *Nv-tra* in diploid embryos from fertilized eggs only (van de Zande and Verhulst, 2014). Additionally, the *Nasonia* female sex-determining mechanism is dependent on maternal provision of *tra* mRNA to the egg, like in many other insect species (e.g. the dipterans *Ceratitis capitata* and *Musca domestica*, and the coleopteran *Tribolium castaneum* (Hediger et al., 2010; Pane et al., 2002; Shukla and Palli, 2012). The roles of *tra* and *dsx* in *Nasonia* sex determination have been described in detail (Oliveira et al., 2009; Verhulst et al., 2010a), but a role of *tra2* within this model has not yet been determined. Here, we describe the structure and splicing of *Nv-tra2* and compare it to other known *tra2* orthologs. We confirm a function of *tra2* in *Nasonia* sex determination through parental RNA interference (pRNAi) experiments and also observed other roles of *Nv-tra2* during development.

2. Material and methods

2.1. Nasonia vitripennis strains and rearing

The *N. vitripennis* lab strain AsymCX (Werren et al., 2010) and the recessive red eye-colour mutant strain ST^{DR} were used throughout the experiments. Homozygous ST^{DR} females mated with AsymCX males produce diploid female offspring with wild-type eyes and red-eyed haploid male offspring). This allows the detection of wild-type diploid males resulting from knockdown of either *Nv-tra2* or *Nv-tra* (Verhulst et al., 2010a). Wasps were reared on *Calliphora* sp. hosts and cultured at 25 °C at a L16:D8 cycle.

2.2. RNA extraction and cDNA synthesis

Total RNA of individual wasps and embryo pools was extracted with TriZol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. RNA was reverse-transcribed with oligo-dT and hexamer primers in a 1:6 ratio with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and stored at −80 °C. RNA samples for gene identification were individual male and female samples of the AsymCX strain. For 3′RACE (Rapid Amplification of cDNA Ends), RNA was reverse-transcribed with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) using 25 μM 3′RACE adapter (5′-GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT 12VN-3′) from FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). For 5′RACE, RNA was processed according to manufacturer's instructions (FirstChoice RLM-RACE kit, Ambion, Austin, TX, USA) and reverse transcribed using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA).

2.3. Identification of Nv-tra2 genomic structure

Primers were designed based on the predicted *Nv-tra2* transcript (LOC100116671) from the *N. vitripennis* genome (Werren et al., 2010) (Table 1). 5′RACE-PCR was performed with outer primer Nvtra2_5Rout and inner primer Nvtra2_5Rin at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, with a final extension of 10 min at 72 °C. Outer primer Nvtra2_3Rout and inner primer Nvtra2_3Rin were used in 3′RACE-PCR with Phusion High-Fidelity DNA polymerase (Fermentas, Hanover, MD, USA). Cycling conditions were 98 °C for 1 min, 35 cycles of 98 °C for 10 s, 57(out)/55(in)°C for 30 s and 72 °C for 60 s, with a final extension of 10 min at 72 °C. Resulting PCR fragments were run and visualized on ethidiumbromide-containing 1.5% agarose gel with 1 × TAE buffer.

All RACE-PCR products were ligated into pGEM-T vector (Promega, Madison, WI, USA) after purification using GeneJET Gel Purification Kit (Fermentas, Hanover, MD, USA). Ligation reactions were used to transform competent JM-109 *Escherichia coli* (Promega, Madison, WI, USA). Colony-PCR was conducted by use of pGEM-T primers (5′-GTA AAA CGA CGG CCA GT-3′) and 5′-GGA AAC AGC TAT GAC CAT G-3′) at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C.

Additional Reverse Transcription-PCRs (RT-PCR) were used to identify splice variants. These PCRs were performed with primers Nvtra2_exon1F/Nvtra2_exon4R and Nvtra2_exon2F/Nvtra2_exon6R at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension of 7 min at 72 °C. PCR-fragments were sequenced on an ABI 3730XL capillary sequencer (Applied Biosystems) and reads were inspected in Chromas (Technelysium) and aligned in MEGA4 (Tamura et al., 2007). Exon-intron structure of the genes was constructed by comparing the mRNA sequences to *N. vitripennis* genome and visualized with Exon-Intron Graphic Maker (http://wormweb.org/exonintron).

2.4. Sequence alignment

Amino acid sequences of TRA2 homologs of the following species (Genbank accession) were used: *Acromyrmex echinatior* (EGI70155), *Apis mellifera* (AFJ15561), *Bombyx mori* (NP_001119705), *Ceratitis capitata* (ACC68674), *Ceratosolen solmsi* (XP_011500657), *Daphnia pulex* (EFX90042), *D. melanogaster* (NP_476764), *Musca domestica* (AAW34233) and *Tribolium castaneum* (AHF71088). Alignments were performed with Geneious8 (Biomatters Ltd).

2.5. Parental RNAi and sample collection

Parental RNAi knockdown was induced in ST^{DR} females in the white pupal stage (Lynch and Desplan, 2006). Non-sex-specific regions were amplified with Nvtra2_RNAiF and Nvtra2_RNAiR primers for *Nv-tra2* dsRNA (564bp) and NvTra_RNAiF1 and NvTra_RNAiR1 primers for *Nv-tra* dsRNA (452bp) (Table 1). The 5′ and 3′ ends of those amplicons were provided with a T7 promotor

Table 1
Overview of primers used in this study. The binding site sequence for T7 RNA polymerase is noted between square brackets.

Primername	Gene	Application	Primer sequence 5′-3′
EF1α_F1[a]	*EF1α*	qRT-PCR	CACTTGATCTACAAATGCGG
EF1α_R1[a]	*EF1α*	qRT-PCR	GAAGTCTCGAATTTCCACAG
NvDsxU_F3[a]	*dsx*	RT-PCR	AGCCACTGCCGAGTATACCA
NvDsxM_R1[a]	*dsx*	RT-PCR	TCGGAGAAGATTGGCAGAAC
NvDsx_qPCR_F1[a]	*dsx*	qRT-PCR	GGTGACATGCGTAGTTTGAG
NvDsx_qPCR_R1[a]	*dsx*	qRT-PCR	CAAGTCGTGGATTTGGTTCG
NvTra_F2[a]	*tra*	RT-PCR	GACCAAAAGAGGCACCAAAA
NvTra_R3[a]	*tra*	RT-PCR	GGCGCTCTTCCACTTCAAT
NvTra_qPCR_F1[a]	*tra*	qRT-PCR	CGCCGTTCTAAGTCATTGAG
NvTra_qPCR_R1[a]	*tra*	qRT-PCR	ATCGGAATAATGCCTATCGT
NvTra_RNAi_F1[a]	*tra*	RNAi	[TAATACGACTCACTATAGGG]CGAGACATCAGTTAGAAGAT
NvTra_RNAi_R1[a]	*tra*	RNAi	[TAATACGACTCACTATAGGG]GTCTTGTGGTCCTATGAAAC
Nvtra2_5Rout[b]	*tra2*	RACE-PCR	GCCCGTTCTGTGATAGAATAATCC
Nvtra2_5Rin[b]	*tra2*	RACE-PCR	GTCCATCAATTGCCATTCCA
Nvtra2_3Rout[b]	*tra2*	RACE-PCR	GTGTACTTTGAATCACTGGA
Nvtra2_3Rin[b]	*tra2*	RACE-PCR	ATTGATGGACGACGAATCAG
Nvtra2_exon1F[b]	*tra2*	RT-PCR	CGCTTTGATTATTATGGACGAC
Nvtra2_exon4R[b]	*tra2*	RT-PCR	GACGGTAGTTTCCTCTGCTG
Nvtra2_exon2F[b]	*tra2*	RT-PCR	CAAGAAGATCAAGAGCCGAC
Nvtra2_exon6R[b]	*tra2*	RT-PCR	CTGAGGTTTGGAATGGTGGA
Nvtra2_qF[b]	*tra2*	qRT-PCR	ACAGAGATAATCCTACTCCAAGCC
Nvtra2_qR[b]	*tra2*	qRT-PCR	CGCTTTGTCTTTGCATCAATGAC
Nvtra2_RNAiF[b]	*tra2*	RNAi	[TAATACGACTCACTATAGGG]CCAAGAAGATCAAGAGCCGA
Nvtra2_RNAiR[b]	*tra2*	RNAi	[TAATACGACTCACTATAGGG]GTCCATCAATTGCCATTCCA

[a] (Verhulst et al., 2010a).
[b] This study.

sequence for dsRNA production using the Megascript RNAi kit (Ambion, Austin, Texas, USA) according to the manufacturer's protocol.

Approximately 600 female ST^{DR} pupae were injected in the abdomen with 1.3 μg/μl of *Nv-tra2* dsRNA, *Nv-tra* dsRNA or, as a negative control, sterile milliQ water. *Nv-tra* was used as a positive control (cf. Verhulst et al., 2010a). Injections were performed with Femtotips II (Eppendorf) needles connected to a Femtojet (Eppendorf, Hamburg, Germany) with continuous injection flow. Hundred and twenty adult females of each category were mated with AsymCX males and evenly divided over two sets for either embryo collections or scoring of offspring production. Virgin females (60 wasps) were set up to produce haploid male controls.

Embryonic levels of *Nv-tra2*, *Nv-tra* and *Nv-dsx* mRNAs and sex-specific splicing of *Nv-tra* and *Nv-dsx* were assessed in early embryos of less than 3 h old. Injected and control females were provided with *Calliphora* sp. hosts in individual egg laying chambers assembled from filter tips (1 ml) capped with a cutoff 1.5 ml vial as a host carrier. The anterior side of the hosts was exposed to the parasitizing female to facilitate localization of the embryos during collection. After 2 h of oviposition in egg-laying chambers, hosts were gently opened to collect approximately 55 embryos in 100% ethanol per sample. Eight embryo samples per category were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Levels and sex-specific splicing of *Nv-tra2*, *Nv-tra* and *Nv-dsx* mRNAs were determined in adult wasps that had been injected with sterile water, *Nv-tra2* or *Nv-tra* dsRNA. Injected females were provided fresh hosts daily to oviposit for 24 h at 25 °C. Five females of each category were individually used in RNA extractions. Parasitized hosts were incubated for 14 days at 25 °C after which offspring emerged. Offspring of *Nv-tra2* and *Nv-tra* injected females were phenotypically screened for sex and eye colour and 10 diploid and 10 haploid males were collected per gene knockdown. Expected sex ratio and eye colour of the offspring were compared to control-group females, and 10 diploid females and 10 haploid males were collected and stored at −80 °C. Of all categories, another two adult offspring were collected and stored at −20 °C for flow cytometry analysis to confirm ploidy (see 2.8).

2.6. mRNA levels in embryos and adults

Quantitative real-time PCR (qPCR) was performed with 5 μl of a 50-fold cDNA dilution and 300 nM PerfeCTaTM SYBR® Green mix (Quanta Biosciences, Gaithersburg, MD, USA) on an Applied Biosystems 7300 Real Time PCR System (Foster City, CA, USA). *Nv-tra2*, *Nv-tra* and *Nv-dsx* were amplified with non-sex-specific, exon-spanning primers at 250 nM (Table 1) (Verhulst et al., 2010a). The *elongation factor 1 alpha* (*EF1α*) was used as a reference gene using exon-spanning primers (250 nM) (Table 1) (Verhulst et al., 2010a). qPCR profiles were 95 °C for 3 min, 45 amplification cycles of 15 s at 95 °C, 58°C/58°C/57°C/55 °C for *Nv-tra2*/*Nv-tra*/*Nv-dsx*/*Nv-EF1α*, 72 °C for 30 s and followed up by a standard ABI7300 dissociation curve. Raw fluorescence data generated by 7300 System SDS Software (Applied Biosystems, Foster City, CA, USA) were base-line corrected and the N0 value calculated for PCR efficiencies per amplicon with LinRegPCR 11.0 (Ramakers et al., 2003). Relative levels were determined by dividing *Nv-tra2*, *Nv-tra* and *Nv-dsx* N0 values by *Nv-EF1α* N0. A one-way ANOVA was used for each life stage (embryo, adult offspring) to test relative differences between the *Nv-tra2*, *Nv-tra* dsRNA injected samples and the water-injected control samples.

2.7. Splice variant analysis of Nv-tra and Nv-dsx following pRNAi

Sex-specific fragments of *Nv-tra* and *Nv-dsx* in injected and control females, and their embryonic and adult offspring, were analyzed by RT-PCR. 5 μl of a 50-fold cDNA dilution was used in a PCR. For sex-specific *Nv-tra* amplification primers NvTra_F2 and NvTra_R3 were used, located at exon 2 and 3, yielding a single 228 bp fragment in females and three fragments of 514, 460 and 282 bp in males depending on their age. Primers NvDsxU_F3 and NvDsxM_R1 were used for sex-specific *Nv-dsx* amplification, yielding 543 bp in females and 651 bp in males (Verhulst et al., 2010a). For amplification and cDNA integrity control *Nv-EF1α* was amplified with NvEF1α_F1 and NvEF1α_R1 primers yielding a 174 bp fragment in both genders. The PCR profile was 45 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Fragments were run and

visualized on an ethidium bromide stained 2% non-denaturing TAE gel.

2.8. Flow cytometry of diploid males

Ploidy of *Nv-tra2* and *Nv-tra* diploid males was confirmed by flow cytometry analysis. Adult male wasp heads were homogenized in Galbraith buffer (21 mM $MgCl_2$, 30 mM tri-sodium citrate hydrate, 20 mM MOPS, 0,1% Triton X-100, 1 mg/l RNAse A) using Dounce homogenizers, filtered by 0.7 μm cell strainer caps (BD Falcon Cell strainer #352235, BD Biosciences, San Jose, California, USA) and stained in propidium iodide (Sigma, St. Louis, Missouri, USA). Samples were loaded on a BD FACS Aria II and analyzed by BD FACSDiva software (BD Biosciences, San Jose, California, USA). References for ploidy were set by haploid and diploid males from a polyploid strain (Beukeboom and Kamping, 2006).

2.9. Viability and fertility of offspring after parental Nv-tra2 and Nv-tra knockdown

Brood sizes were analyzed to assess the survival of offspring from *Nv-tra2* dsRNA, *Nv-tra* dsRNA, sterile water injected and non-injected mothers. The number of embryos was compared to the number of emerging adult offspring. ST^{DR} female pupae were injected with dsRNA targeting *Nv-tra2* (N = 200), *Nv-tra* (N = 200) and sterile water (N = 200). Of each category 100 adult females were mated with AsymCX males, while the other 100 remained virgin. Mated and virgin females were individually cultured in egg laying chambers and divided over 2 batches which were alternately used for embryo or adult offspring counts. This prevented biases caused by knockdown efficiency or fecundity of the wasps. It additionally allowed exclusion of females that were not mated and females that were not injected from further analysis. Wasps were allowed to parasitize on the anterior side of the hosts for 2 h at 25 °C, which was repeated on four consecutive days at a similar time of day. Females were allowed to oviposit for 2 h, after which they were removed and hosts were either opened for embryo counts or retained at 25 °C for 14 days to count adult offspring. Numbers of embryos and adult offspring were compared in a general linear model and a post-hoc Tukey-test was used to examine sample differences in embryo and adult number. Differences in egg number laid by females in each category were compared with a Kruskal-Wallis test.

Adult male offspring from these experiments were used to assess fertility of haploid and diploid *Nv-tra2* and *Nv-tra* pRNAi males compared to control males (haploid males from water-injected mothers). Each male (N = 20 per category) was mated to 7 virgin females very shortly after one another to induce sperm depletion (Ruther et al., 2009). These females were hosted on 4 *Calliphora* sp. pupae every other day over a period of 10 days (5 data points). Offspring were scored after emergence and the fraction of data points (of 7 females x 5 rehostings) producing daughters was calculated for each male. This provided a measurement of (diploid) male fertility. Differences between categories were compared with a one-way ANOVA.

3. Results

3.1. Structure, splicing and conservation of tra2 in N. vitripennis

To identify a *tra2* homolog in *N. vitripennis*, the reference genome of AsymCX (Werren et al., 2010) was screened with an *A. mellifera* TRA2 query (Genbank accession AFJ15561) using tblastn. A putative homolog of *tra2* (Genbank accession XP_001601106, predicted isoform X3) could be detected that had previously been described in an amino acid comparison in Nissen et al. (2012). This predicted sequence was used for RACE-PCR and RT-PCR primer design to identify the gene structure. Four splice variants of *Nv-tra2* were detected in *N. vitripennis* based on a combination of RACE-PCRs and verified by RT-PCRs (Fig. 1). The most commonly detected variant was $Nv\text{-}tra2^A$. The less abundant variants $Nv\text{-}tra2^B$ and $Nv\text{-}tra2^C$ were identified by their coding region corresponding to exon 5 and 6a (Fig. 1). Variant $Nv\text{-}tra2^D$ was detected in low quantities, includes exon 3 and leads to a truncated ORF. An additional 24 bp (5′-CATCATTTGCTACCTTACACACAG-3′) was present in very low frequencies in one or more splice variants, including the 5′ end of exon 2. Splice variant $Nv\text{-}tra2^A$ matches exactly with the predicted isoform X3 (Genbank accession XP_001601106). Only splice variants $Nv\text{-}tra2^C$ and $Nv\text{-}tra2^D$ were not predicted by the automated computational analysis of the *tra2* locus (NCBI *Nasonia vitripennis* Annotation Release 101). No sex-specific splice variants were detected. All splice variants are deposited in Genbank with accession number: KY938035 ($Nv\text{-}tra2^A$); KY938036 ($Nv\text{-}tra2^B$); KY938037 ($Nv\text{-}tra2^C$); KY938038 ($Nvtra2^D$).

The structure of TRA2, consisting of a single RNA-binding domain (RBD) flanked by RS-rich regions, is conserved in *N. vitripennis*. Unlike in *A. mellifera* (Nissen et al., 2012) no variation was found in the length of the first RS-rich domain. The 24 bp stretch is just upstream of this region. The second RS-rich region is entirely absent in the low abundant splice variants C and D. Variant $Nv\text{-}Tra2^D$ lacks a large part of the RBD region, which is fully present in the other splice variants (Fig. 1).

The amino acid sequence of NV-TRA2 was aligned to known TRA2 homologs, revealing strong conservation, in particular within the Hymenoptera (Fig. 2). A notable feature, compared to other insect TRA2 peptides, is the presence of a glycine-rich region at the 3' end of the $Nv\text{-}tra2^A$, $Nv\text{-}tra2^B$ and $Nv\text{-}tra2^C$ splice variants (presence in $Nv\text{-}tra2^A$ depicted in Fig. 2 between positions 266 to 288). This glycine-rich region is present in *D. melanogaster* RBP1 which is an important co-factor in the TRA-TRA2 regulation of *dsx* splicing (Heinrichs and Baker, 1997). The Gly-rich domain in RBP1 is directly involved in protein-protein interactions between RBP1 and TRA2. It is also present in the chalcid *Ceratosolen solmsi* (XP_011500657 (Xiao et al., 2013)) and *B. mori* (Niu et al., 2005) (Fig. 2), which could signify a gain of TRA2 function in these systems.

3.2. Sex reversal effects of Nv-tra2 parental RNAi knockdown

A pilot RT-PCR experiment revealed *Nv-tra2* presence in early embryos (<3 h old) indicating maternal provision of *Nv-tra2* to the eggs (original data not shown, but the presence of maternally provided *Nv-tra2* is depicted here in Fig. 3a control and *Nv-tra* pRNAi samples). The bulk of zygotic expression in embryos laid by oviparous animals is initiated at the midblastula transition, a precise developmental point prior to gastrulation (Langley et al., 2014). In *N. vitripennis* blastoderm formation occurs 4–9 h after egg laying after which gastrulation starts (Bull, 1982), indicating that the midblastula transition in *N. vitripennis* starts no earlier than 4 h after egg laying. We assume this 4 h time point as the start of large-scale zygotic transcription in *N. vitripennis* and have shown that zygotic transcription of *Nv-tra* starts at 5 h of development in embryos from fertilized eggs (Verhulst et al., 2010a). As all embryonic samples in this study were less than 3 h of age, the *Nv-tra* transcripts have a maternal origin (Verhulst et al., 2010a) and the *Nv-tra2* transcripts are also considered to be of maternal origin. By using pRNAi it is then possible to knockdown the maternal provision of *Nv-tra2* mRNA to the eggs. In order to target this maternal provision of all *Nv-tra2* splice variants, a 565 bp sequence

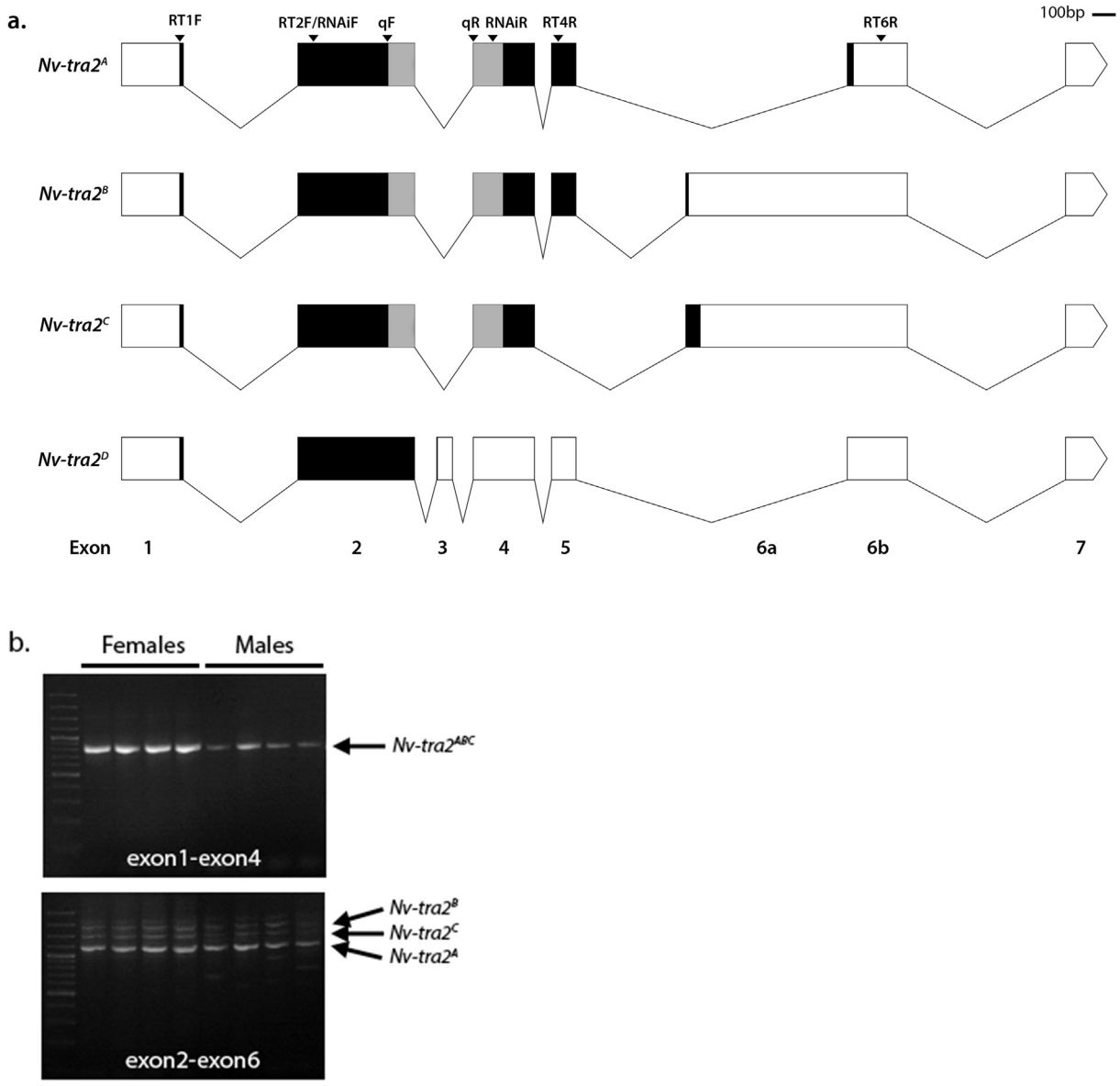

Fig. 1. Genomic structure of *Nv-tra2* (a). Blocks represent exons, numbered at the bottom of the figure, and lines represent the introns. The white regions depict the 5′UTR and 3′UTR, and the black exons depict the coding region. The 24bp 5′ addition to exon 2 is not shown here. The RBD domain is plotted in grey on the exons, only when present in full. Positions of primers used to generate the dsRNA construct (RNAi), to depict splicing patterns (RT) and to measure the *tra2* expression (q) are indicated at the top. The scale bar depicts 100 base pairs. Splicing patterns of *Nv-tra2* in adult female and male wasps (b). Each slot contains the amplified RT-PCR fragments of one individual. *Nv-tra2*^{D} is very rare and not visible on the top gel. *Nv-tra2*^{A}, *Nv-tra2*^{B} and *Nv-tra2*^{C} share exons 1 till 4. The splice variation in exon 5 and 6a is depicted on the bottom gel.

containing the RBD and spanning all splice variants was used to synthesize dsRNA (primer positions shown in Fig. 1). This *Nv-tra2* dsRNA was injected into females in the pupal stage. RNAi off-target effects were predicted with WaspAtlas (Davies and Tauber, 2015) and no targets other than *Nv-tra2* were detected. Parental knockdown (pRNAi) with *Nv-tra2* dsRNA led to a 10-fold reduction of *Nv-tra2* transcripts in the offspring compared to that of water injected females ($F_{(2,15)} = 27.1$, $P < 0.001$) (Fig. 3a), but no effect on the level of *Nv-tra* mRNA (Tukey-test: $P = 0.22$) (Fig. 3b). Parental knockdown of *Nv-tra* led to a significant decrease in *Nv-tra* mRNA levels in the offspring compared to that of water-injected females ($F_{(2,15)} = 13.3$, $P < 0.001$) (Fig. 3b), but no significant effect on *Nv-tra2* mRNA levels (Tukey-test: $P = 0.18$) (Fig. 3a). Hence, pRNAi of either gene does not interfere with the maternal transcript provision of the other.

Adult offspring of *Nv-tra2* knockdown mated females were scored after emergence; they contained no daughters but consisted solely of a modest number of diploid males and a larger number of haploid males (Table 2). Mated females injected with water produced diploid female and haploid male offspring as expected under haplodiploidy, indicating that the parental knockdown of *Nv-tra2* transcripts in females caused a sex reversal of their diploid offspring, turning them into males whereas haploid offspring were unaffected. *Nv-tra* pRNAi also resulted in diploid and haploid male offspring, in agreement with earlier observations (Verhulst et al., 2010a). The ploidy of males with wild-type and red eye colour was confirmed with flow cytometry to be diploid and haploid, respectively. The water-injected mated females produced progeny with a low sex ratio (calculated as the number of males divided by the total number of offspring). In the adult offspring of injected

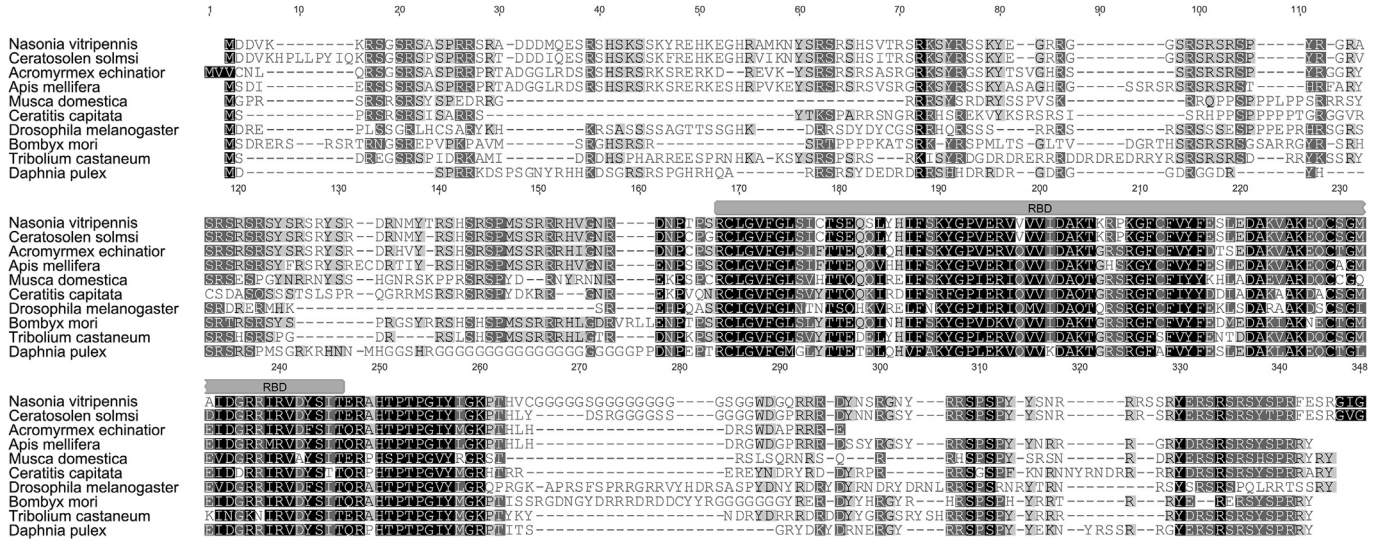

Fig. 2. Alignment of TRA2 amino acid sequences. The RBD domain is marked in grey above the sequences. Conservation of sites is depicted in grey scale, with darker colours indicating higher conservation, and lighter colours indicating lower conservation. The Gly-rich region is positioned from amino acid position 266–288 in *N. vitripennis*; from 274 to 288 aa in *C. solmsi*, and from 267 to 290 aa in *B. mori*.

mothers, relative levels of *Nv-tra2* and *Nv-tra* mRNA had recovered, after the initial reduction in early embryonic stages. The sex-specific splicing of sex determination genes *Nv-tra* and *Nv-dsx* was assessed in the adult offspring of *Nv-tra2* dsRNA-, *Nv-tra* dsRNA- and water-injected females. Diploid females and haploid males from water-injected mothers have normal female- or male-specific splicing of *Nv-tra* and *Nv-dsx* as expected. Both *Nv-tra2* and *Nv-tra* dsRNA-injected mated females produced an all-male progeny, consisting of haploid and diploid males, that all showed splicing of *Nv-tra* and *Nv-dsx* in the male-specific mode (Fig. 4). This suggests that the role of *Nv-tra2* in the sex determination cascade is conserved at the level of interaction with *Nv-tra*. Furthermore, both *Nv-tra* and *Nv-tra2* transcripts are detected prior to the presumed start of zygotic transcription around 4 h after egg laying. This early presence is presumed to result from maternal provision of the *Nv-tra* and *Nv-tra2* transcripts to the zygote and required for female development of fertilized eggs.

3.3. Functionality of Nv-tra2: viability and additional knockdown effects

All diploid individuals observed after *Nv-tra2* knockdown were males, but their number was very low compared to the number of diploid males after *Nv-tra* knockdown and of diploid females after injections with water (Table 2). This reduction in offspring after pRNAi could result from a reduced oviposition rate or from lethality during development. To determine which was the case, the number of eggs and adult offspring produced by mated and virgin females were compared. A large discrepancy between the number of oviposited eggs and emerging adults, in absence of larval or pupal remains, would indicate embryonic inviability, whereas equally lowered numbers of eggs and adults would indicate reduced oviposition.

In *Nv-tra2* pRNAi virgin females, a significant difference was detected between the number of oviposited eggs and the number of emerged offspring ($F_{(4,1014)} = 9.8$, $P < 0.001$, Tukey-test: $P < 0.001$). This result indicates an effect of *Nv-tra2* on offspring viability. As no dead larvae or pupae were observed in opened hosts, this lethal effect appears to occur during embryonic development. The number of eggs deposited by *Nv-tra* pRNAi virgin females did not significantly differ from the number of emerged offspring (Tukey-test: $P = 0.74$) (Fig. 5a). Mated females injected with either *Nv-tra2* (Tukey-test: $P < 0.001$) or *Nv-tra* dsRNA (Tukey-test: $P < 0.001$) showed a discrepancy between number of oviposited eggs and adult emergence ($F_{(4,971)} = 34.9$, $P < 0.001$) (Fig. 5b), indicating embryonic lethality in both dsRNA treatments. Overall, fewer embryos were found for both *Nv-tra* and *Nv-tra2* dsRNA-injected females ($H_{(3,507)} = 84.7$, $P < 0.001$) (Fig. 5a) when compared to non-injected females, but this reduction was also present in the water-injected control category, suggesting an effect of handling and injection trauma more so than potential dsRNA side effects.

Since *Nv-tra2* pRNAi caused mortality in the offspring of the treated wasps, the fitness of the surviving male offspring was assessed by testing their fertility. This showed that haploid and diploid *Nv-tra* pRNAi male offspring as well as haploid and diploid *Nv-tra2* pRNAi male offspring had normal capabilities to fertilize females compared to the control haploid male offspring of water-injected mothers (Fig. 6). A significant difference was only found between haploid and diploid *Nv-tra* pRNAi male offspring ($F_{(4,94)} = 3.5916$, $P = 0.009$, Tukey: $P = 0.003$). As neither haploid nor diploid *Nv-tra* pRNAi male offspring differed in fertilization abilities from the haploid control male offspring, we suggest that this significant difference is an effect of the small sample size used in this experiment ($N = 20$ per category) or an effect of experimental procedure caused by slight timing differences in collection of males and set-up of matings.

4. Discussion

4.1. Conservation of tra2 in the N. vitripennis sex determination cascade

We showed that an ortholog of *tra2* is present in the genome of *N. vitripennis*, and that it is not sex-specifically spliced. This is corroborated by observations of *tra2* splicing in other insects (Martín et al., 2011; Nissen et al., 2012; Niu et al., 2005; Shukla and Palli, 2013). The *tra2* gene is an important component of the *Nasonia* sex-determining cascade, as prevention of *Nv-tra2* maternal transcript provision by pRNAi leads to male-specific splicing of both *Nv-tra* and *Nv-dsx* pre-mRNA in diploid fertilized

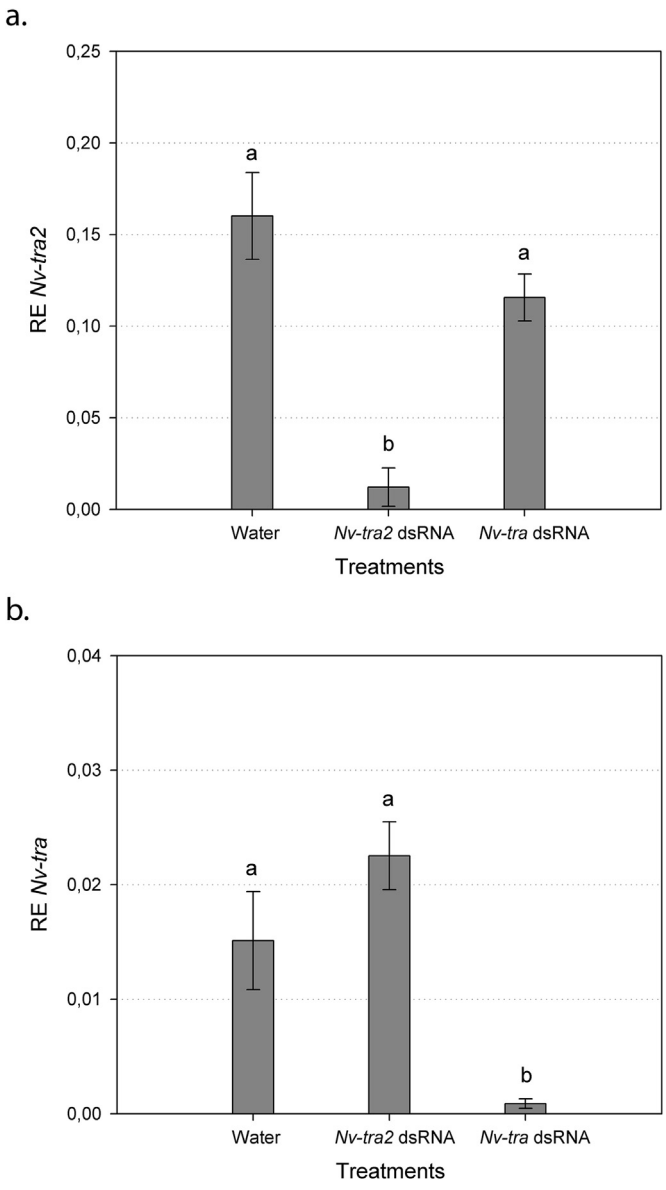

Fig. 3. Relative expression (RE) in embryos (0–3 h) prior to zygotic transcription. Relative levels of *Nv-tra2* mRNA (a) and *Nv-tra* mRNA (b) in control embryos (water), embryos of *Nv-tra2* pRNAi females and embryos of *Nv-tra* pRNAi females. Different letters above the bars indicate significant differences between treatment groups (Tukey test: $P < 0.05$).

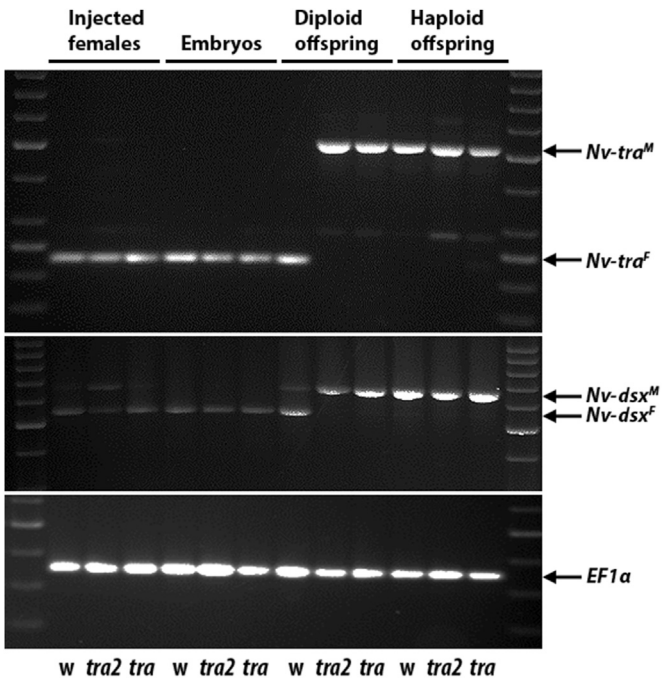

Fig. 4. Sex-specific splicing patterns of *Nv-tra* and *Nv-dsx* in pRNAi females and offspring. Amplicons are produced by RT-PCRs on pooled samples of females (five samples containing one female each), embryos (0–3 h) (eight samples of ~55 embryos) and diploid (2N) and haploid (N) adult offspring (one day after emergence; ten samples containing one individual each). The injected substance (water, *Nv-tra2* dsRNA or *Nv-tra* dsRNA) is depicted underneath each sample. Arrows mark female-specific splicing and male-specific splicing of *Nv-tra* and *Nv-dsx*. Control gene *EF1α* is displayed at the bottom.

eggs and subsequent differentiation into functional males.

The sex reversal from female to male development of fertilized eggs upon parental knockdown of *Nv-tra2* expression indicates functional and positional conservation of *tra2* in the *Nasonia* sex determination cascade. Absence of *Nv-tra2* mRNA in early embryonic stages fails to direct the sex determining pathway of diploid embryos towards female development, as only male-specific splice variants of *Nv-tra* and *Nv-dsx* are detected. Although it is possible that pRNAi also reduces zygotic transcripts in later embryonic stages, our results indicate that maternally provided transcripts are crucial for a timely activation of female sex determination in *N. vitripennis*. This is supported by the fact that in both the CSD mechanism of *A. mellifera* (Gempe et al., 2009) and the sex determination mechanism in the hymenopteran *A. tabida* (Geuverink, unpublished data) maternal provision of *fem* or female-specific *tra* mRNA is absent and female sex determination ensues differently. Our results also indicate that NV-TRA2 is required for initiation of the female-specific *Nv-tra* auto-regulatory loop. After all, if NV-TRA2 would only interact with *Nv-dsx*, maintenance of female-specific splicing of *Nv-tra* would be unaffected and only male-specific splicing of *Nv-ds*x would occur. Similar to *A. mellifera* (Nissen et al., 2012), in *Nasonia* TRA2 acts on two levels in the sex determination cascade, regulating the splicing of both *tra* and *dsx*.

4.2. Role of Nv-tra2 in embryo viability

Following pRNAi of *Nv-tra2*, a high lethality of both haploid and diploid offspring was observed, while *Nv-tra* pRNAi led to only high lethality in the diploid offspring. This suggests that (1) *Nv-tra2* has

Table 2
Offspring number, ploidy and sex of *Nv-tra2* dsRNA, *Nv-tra* dsRNA and water-injected females. Numbers of tested females (P: females (RNAi)) and counts of their offspring (F1: haploid males, F1: diploid females and F1: diploid males) are shown. Mean numbers of haploid and diploid offspring per female are based on these counts.

Treatment	P: ♀(RNAi)	F1: haploid ♂	F1: diploid ♀	F1: diploid ♂	F1: diapause	Mean number of haploid offspring	Mean number of diploid offspring
Nv-tra2 injected	61	251	0	46	83	4.11	0.75
Nv-tra injected	56	212	0	450	711	3.79	8.04
Water control	55	210	1195	0	0	3.82	21.73

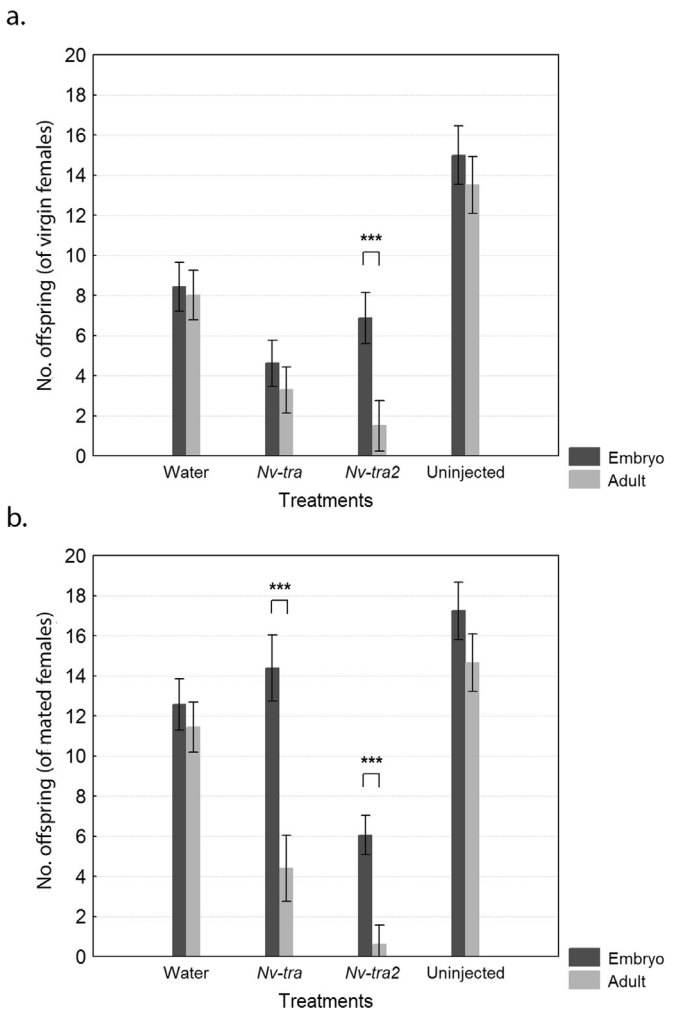

Fig. 5. Offspring counts of (a) virgin and (b) mated females. Virgin females produce only haploid offspring and mated females produce a majority of diploid offspring when hosted individually. The number of eggs laid in a host is plotted in dark grey and the number of adult offspring which emerged from a host is plotted in light grey. The stars indicate the level of significance between the number of embryos and adults of the same treatment: ***P < 0.001.

other functions in early development, as lowered maternal *Nv-tra2* provision leads to inviable haploid offspring and (2) the lack of *Nv-tra2*, and possibly also *Nv-tra*, maternal mRNA impairs the development of the diploid zygote (Fig. 7). Unfortunately, we cannot distinguish between the causes of the observed inviable offspring and diploid lethality after *Nv-tra2* knockdown. Our first conclusion is corroborated by Nissen et al. (2012) who reported effects of *tra2* knockdown on embryogenesis in *A. mellifera* that were not sex-specific and independent of *tra* regulation. This suggests a similar role of *Nv-tra2* in embryogenesis as in *A. mellifera*, but our experimental approach differs from Nissen et al. (2012). Our pRNAi blocks maternal provision, whereas in *A. mellifera* dsRNA is injected directly in embryos to silence zygotic transcription. In dipterans and lepidopterans, RNAi studies have not shown any function for *tra2* in embryogenesis (Burghardt et al., 2005; Salvemini et al., 2009; Suzuki et al., 2012). However, in *T. castaneum* (Shukla and Palli, 2013) RNAi with *tra2* dsRNA at the larval stage led to developmental arrest, suggesting that *tra2* could have acquired additional developmental functions in Hymenoptera and Coleoptera or has lost such functionality in Diptera and Lepidoptera.

Our second conclusion may be caused by an additional mechanism. A decreased survival of diploid offspring following *Nv-tra* pRNAi can also be seen in the data of Verhulst et al. (2010a) where mated mothers produced 44% haploid offspring. This deviates from a normal progeny sex ratio of a single foundress that typically contains about 15% haploid (male) offspring, as observed in the progeny sex ratio of the water injected females of this study. *Nv-tra* pRNAi does not seem to impact haploid offspring that, under normal developmental conditions, would not produce a functional TRA protein. Viable diploid males are observed in a polyploid mutant *N. vitripennis* strain (Whiting, 1960), however, these individuals emerge from unfertilized diploid eggs, and thus do not carry a paternal genome set (Beukeboom et al., 2007). In this study, diploid offspring in both pRNAi classes differ from their haploid brothers in obtaining a paternal chromosome set upon fertilization, hence they are biparental. One possible explanation for the higher mortality of these diploid embryos resulting from *Nv-tra* or *Nv-tra2* pRNAi may be that the absence of the TRA-TRA2 complex is incompatible with the activated genes from the paternal genome, which would then lead to an early developmental arrest, but further research is necessary to understand the cause of the mortality.

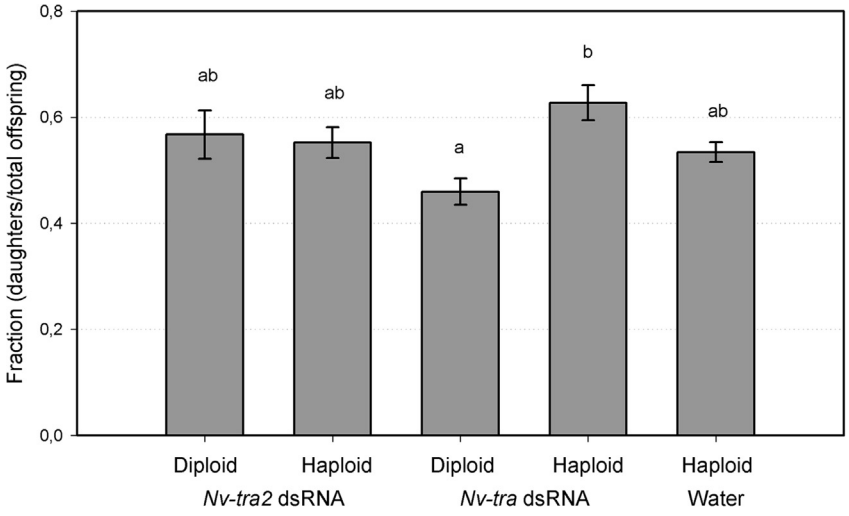

Fig. 6. Fertility of knockdown male offspring. Mean fraction of daughters/total offspring per vial produced by haploid and diploid males of *Nv-tra2* pRNAi, *Nv-tra* pRNAi and water-injected females. Different letters above the bars indicate significant differences between categories (p < 0.05).

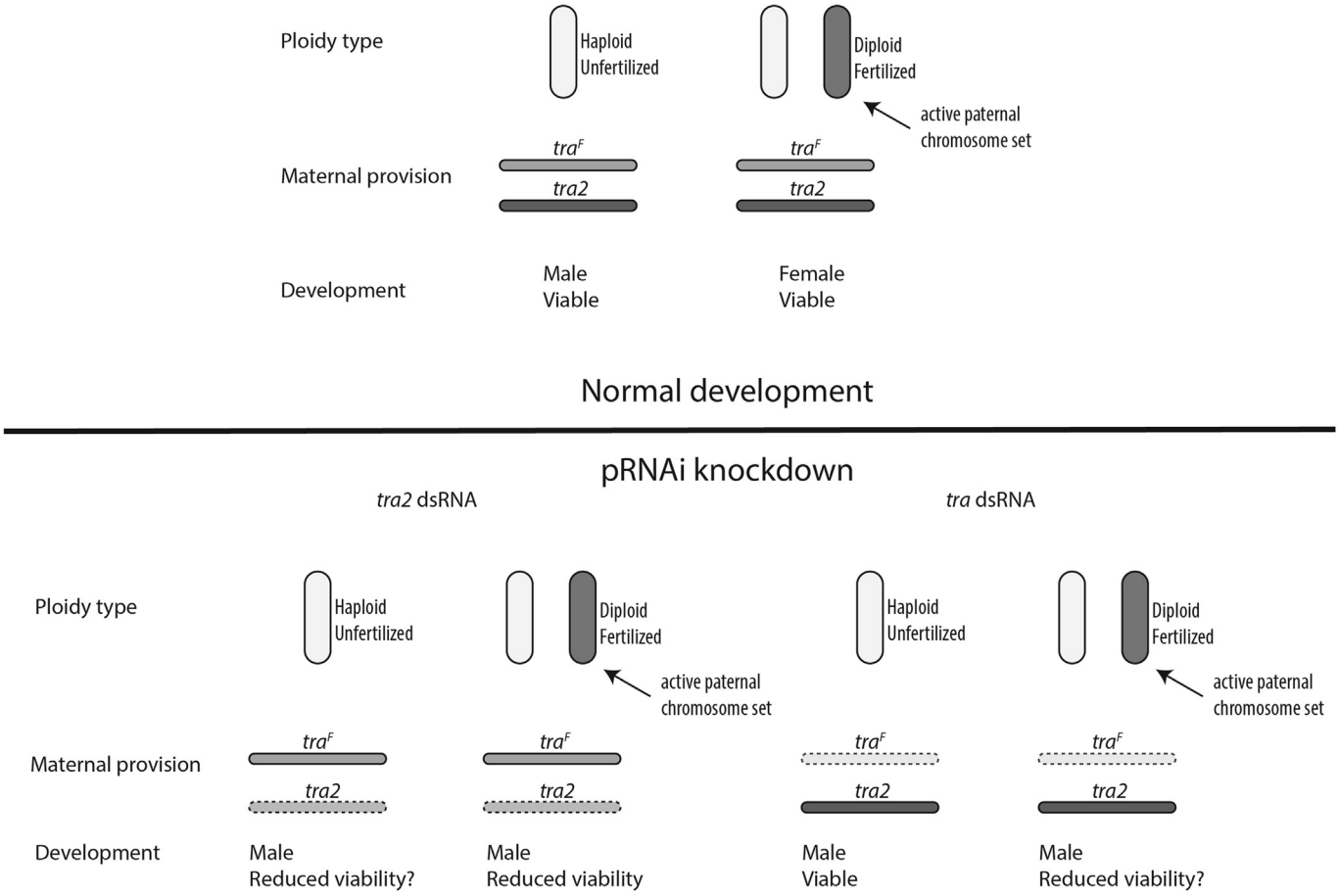

Fig. 7. Overview of maternal provision and resulting offspring development in non-manipulated and pRNAi *N. vitripennis* wasps. Depicted at the top is the normal embryonic development of *N. vitripennis;* at the bottom is the embryonic development following pRNAi with *Nv-tra* or *Nv-tra2* dsRNA. The ploidy of the embryo is represented by one or two chromosomes, with the maternal chromosome in light grey and the paternal chromosome in dark grey. Maternal provision is indicated by horizontal bars representing mRNA of *Nv-tra* (tra^F) and *Nv-tra2 (tra2).* Knockdown of maternal provision by pRNAi is portrayed with a dotted outline, while the unaffected maternal provision is indicated with a solid outline. Underneath, the resulting sexual development is stated with the observed viability.

4.3. Surviving diploid males do not differ from haploids in splicing and fertility

Despite the reduced viability of diploid *Nv-tra* pRNAi male offspring and an almost complete inviability of diploid *Nv-tra2* pRNAi male offspring, the surviving males do not appear to suffer subsequent effects. Their sex determination is firmly fixed in the male mode with male-specific splicing of both *Nv-tra* and *Nv-dsx*. Furthermore, their ability to mate with females and fertilize their eggs does not appear to be impacted. This suggests that only very early processes, possibly at the start of zygotic transcription, result in the observed inviability. Once the male developmental pathway is firmly established, these males appear not to encounter any further costs of their diploid status.

4.4. Activation of tra under CSD and MEGISD

The requirement of maternally provided *Nv-tra* and *Nv-tra2* for survival of diploid embryos highlights the importance of maternal effects in the *N. vitripennis* MEGISD sex determination (Verhulst et al., 2010a). Maternal provision of *Nv-tra* alone is not sufficient to start the female-specific cascade; *Nv-tra2* mRNA is required in the early embryo as well to ensure both female development and proper embryonic development. In the honeybee, RNAi of *fem* does not lead to noticeable mortality (Beye et al., 2003; Gempe et al., 2009; Hasselmann et al., 2008) and mortality after *tra2* embryonic RNAi is independent of ploidy (Nissen et al., 2012). Diploid males resulting from homozygous *csd* alleles can develop in *A. mellifera*, but are killed by workers before reaching adulthood (Woyke, 1963). Apparently, silencing this transducing stage of the sex determination cascade has different consequences for the CSD mechanism of *A. mellifera* than for the MEGISD mechanism of *N. vitripennis*. Fem^F in *A. mellifera* is not maternally provided contrary to the tra^F mRNA provision in *N. vitripennis* (Gempe et al., 2009; Verhulst et al., 2010a). The female-specific cascade in *A. mellifera* is activated in the presence of two different *csd* alleles. *N. vitripennis* sex determination relies on a silencing mechanism in the mother, or an activating signal in the father. These differently imprinted chromosome sets may result in additional detrimental effects when present in an embryo developing into the opposite sex.

Acknowledgements

We thank Charlotte Capt for performing a pilot *Nv-tra2* pRNAi study and the entire Evolutionary Genetics group for proof-reading this manuscript. We thank two anonymous reviewers for their valuable comments and suggestions.

This work was supported by NWO TOP grant [no. 854.10.001] to LWB and NWO Veni grant [no. 863.13.014] to ECV.

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